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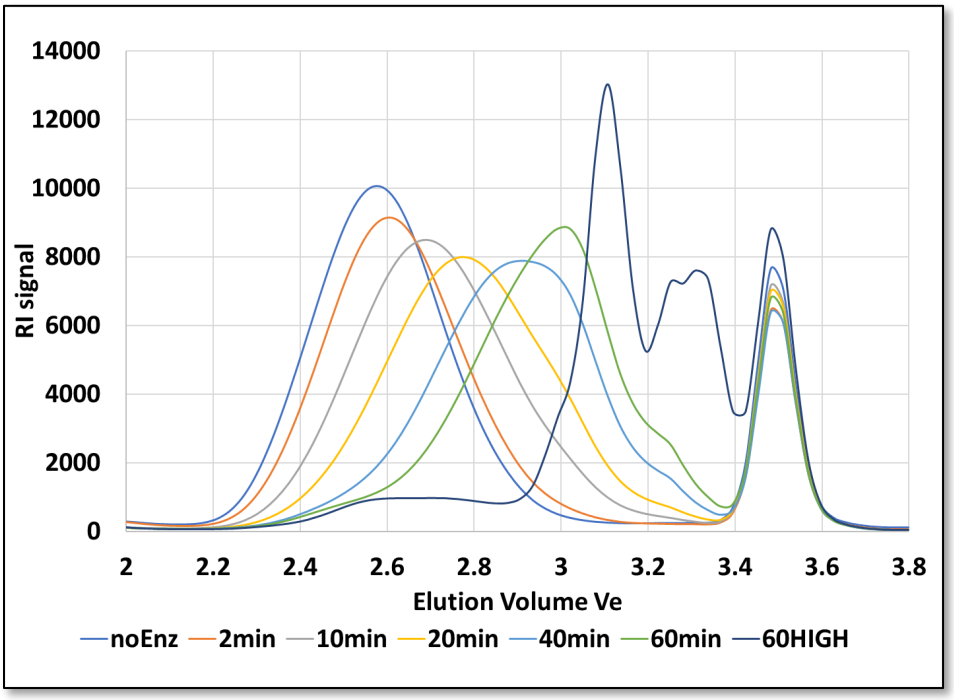
Development of a High-Performance Gel Filtration Chromatography Polysaccharide Sizing Method to Facilitate Glycoside Hydrolase Functional Analysis

Lisa M. Koch
Casey Crooks
Franz J. St John



Abstract

Gel filtration chromatography (GFC) is a form of aqueous liquid chromatography intended for separation of molecules based on effective molecular volume. With the advent of high-pressure liquid chromatography (HPLC) systems, liquid chromatography techniques have greatly improved. Further improvements have occurred with the development of more rigid and uniform stationary phase resins. GFC applications that employ modern liquid chromatography instrumentation and columns are often dubbed as "high-performance" (HPGFC). Given a need to study the process of enzymatic hemicellulose degradation, we determined that an HPGFC method would be beneficial to develop for use by the Forest Products Laboratory. In this report, we describe a laboratory method of HPGFC for the high-resolution separation of soluble hemicellulosic polysaccharides. Using this method, we can gain greater understanding of how xylan- and mannan-processing enzymes function.

Keywords: HPGFC, gel filtration chromatography, size exclusion chromatography, gel permeation chromatography, xylan, hemicellulose, enzyme processing

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USDA Forest Service, Forest Products Laboratory
One Gifford Pinchot Drive, Madison, WI, USA 53726
(608) 231-9200
SM.FS.MailroomFPL@usda.gov

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Lisa M. Koch, Biochemist
Casey Crooks, Research Microbiologist
Franz J. St John, Research Microbiologist
USDA Forest Service, Forest Products Laboratory, Institute for Microbial and Biochemical Technology, Madison, Wisconsin, USA

Introduction

Size exclusion chromatography (SEC) is a form of liquid chromatography that separates molecules based on their size (Lathe and Ruthven 1956, Moore 1964, Barth 2013, Ó'Fágáin et al. 2017). Like all liquid chromatography applications, analytes (such as molecules and proteins) are injected into a mobile phase as it enters one end of a column filled with a mobile-phase-equilibrated solid-phase matrix or resin. The resin consists of inert porous beads. Beads can measure from single digits to tens of microns, and the pores within the beads from 10 nm to greater than 100 nm. The specific bead and pore sizes define the fractionation range of any given column system. Analytes traveling with the mobile phase through the column differentially interact with the pores based on size. For molecules sized within the defined fractionation range, the smaller molecules will interact with the porous structure of the beads more often and the larger molecules less often. In so doing, the smaller molecules will traverse through the column more slowly and elute later than the larger molecules, which interact with the pores less, thus allowing them to elute earlier.

Size exclusion chromatography has been divided into two general categories: (1) aqueous SEC, which is referred to as gel filtration chromatography (GFC) or molecular sieve chromatography, and (2) nonaqueous, organic mobile phase SEC, referred to as gel permeation chromatography (GPC). Other than the primary mobile phase difference, these two techniques are identical in how they function to separate molecules based on size. The choice of which to utilize is primarily one of comparative analyte solubility in aqueous or organic solvents.

Hemicelluloses constitute the second most abundant class of polysaccharides on earth. They are primarily represented by either a form of β-1,4-linked repeating xylose or a β-1,4-linked glucomannan. These are the major forms of hemicellulose in woody biomass (Ebringerová et al. 2005). Currently, intense interest is being focused on identifying the best use of these polysaccharides. In the simplest use, these polysaccharides can be hydrolyzed to monosaccharides and converted through microbial or enzymatic bioconversion to valuable chemicals or green fuels or chemically converted to bioadvantaged compounds such as furfural or 5-hydroxymethylfurfural (Carvalheiro et al. 2008). Other uses include diet supplementation of oligomeric forms of these polymers as prebiotics to promote human or farm animal health and well-being (Granato et al. 2022). Polymeric forms of these hemicellulosic polysaccharides may be used in the development of new materials through cross-linking and further modification. Thin films, oxygen barrier films, and hydrogels are all being considered as potential products made from these naturally derived abundant polysaccharides (Encina et al. 2021, Giri and Ghosh 2021, Sun et al. 2021).

Efficient utilization of these hemicelluloses requires enzymes that depolymerize or process these polysaccharides to generate desired products. For xylan, enzymes such as endoxylanases, xylosidases, arabinofuranosidases, and α-glucuronidases as well as other unique activities are involved in its degradation. When studying xylanase enzyme treatment of polymeric xylan, it is sometimes important to consider not only what small products are being generated but also what changes are occurring to the actual polysaccharide. One way to do this is by considering the change in molecular weight of the polymer in addition to the simultaneous release of small sugars (Eremeeva and Bykova 1993, Jacobs and Dahlman 2001, Pitkänen et al. 2017).

In this paper we report on the development of a high-performance gel filtration chromatography (HPGFC) method for the study of hemicellulosic polysaccharides and the specific glycoside hydrolase enzymes that act to degrade these carbohydrate polymers. Using a size-resolved fraction of soluble beechwood glucuronoxylan (BWX), the method

is modeled by demonstrating how an endo-acting (internal cleaving) GH10 endoxylanase processes the polymeric xylan compared with an exo-acting (end cleaving) GH30-10 xylobiohydrolase. In future studies this method will be used to measure trace endo activity within a group of closely related xylobiohydrolases and to test endoxylanases for a functional characteristic referred to as processivity.

Materials and Methods

Reagents

All reagents were of the required purity level. Pullulan molecular weight standards were purchased from Sigma-Aldrich (Saint Louis, Missouri) and Shodex (Resonac America, New York, New York). Beechwood glucuronoxylan was purchased from Sigma-Aldrich and prepared to 20 mg/mL as previously described (St John et al. 2018). This preparation was further improved for sizing studies by chromatographic fractionation. Xylose for use as a standard was purchased from Absolute Standards Inc. (Hamden, Connecticut). The endoxylanase *Pb*Xyn10A-CD (*Pb*Xyn10A) (St John et al. 2006, 2012) and the exo-acting nonreducing terminal xylobiohydrolase *Ac*Xbh30A (Crooks et al. 2021, St John et al. 2022) were used from –80 °C frozen stocks that were prepared in the development of original research.

Biochemical Studies

Total carbohydrate was measured using the phenol-sulfuric total carbohydrate assay (Dubois et al. 1956) using xylose to generate a standard curve.

Automated Chromatography Systems

Initial chromatographic studies and the fractionation of BWX utilized a Bio-Rad BioLogic Duo-Flow medium-pressure chromatography system (FPLC) equipped with a QuadTec UV-VIS multiple wavelength detector (MWD) (Bio-Rad Laboratories, Inc., Hercules, California) and a Jasco RI-2031+ refractive index detector (RID) (Jasco, Inc., Easton, Maryland). For more analytical results, development of the HPGFC method was performed using an Agilent 1260 Infinity (Agilent Technologies, Santa Clara, California) high-performance liquid chromatography (HPLC) system equipped with a RID. The system was controlled, data acquired, and data managed using the Agilent OpenLab software.

High-Performance Gel Filtration Chromatography

Using an Agilent 1260 Infinity HPLC system, HPGFC was performed; chromatographic separation was achieved with a TSKgel SuperMultipore PW-N (part# 22789, 6.0 mm ID × 15 cm long, 4 um spherical particles; Tosoh Biosciences, King of Prussia, Pennsylvania) in combination with the recommended guard column (part #22793, 4.6 mm ID × 3.5 cm long). Column and guard column total volume was calculated to be 4.82 mL. The mobile phase or gel filtration buffer (GFB) selected for this method consisted of 150 mM sodium chloride, 50 mM sodium acetate, pH 5.0. Flow rate was maintained at 0.4 mL/min, and the column was maintained at 45 °C. Sample volumes of 10 µL were injected, and RID data were collected. The HPGFC system was calibrated using pullulan molecular weight standards.

Preparation of a Beechwood Glucuronoxylan Substrate

FPLC was performed with a Sephacryl S-200HR column in a water mobile phase at 1.5 mL/min for 320 mL. Fractions resulting from six 5-mL injections of 20 mg/mL BWX were collected, combined, and analyzed for total carbohydrate and by HPGFC.

Beechwood Glucuronoxylan Enzyme Treatments

*Pb*Xyn10A and *Ac*Xbh30A were used at 0.5 µg/mL in 60 µL reactions with 2.5 mg/mL BWX fraction H for 2, 10, 20, 40, and 60 min. No enzyme, no substrate, and 50 µg/mL "high enzyme" reactions were also run for 60 min. All reactions were completed in mobile phase (100 mM NaCl, 50 mM sodium acetate pH 5.0) for convenience. *Ac*Xbh30A reactions were run at 60 °C with 0.02 mg/mL BSA. *Pb*Xyn10A reactions were run at 45 °C. Reactions were stopped by heating to 95 °C for 5 min followed by ice for 5 min and a 16k × *g* spin for 5 min to remove particulate from samples before loading on the HPLC.

Results and Discussion

Calibrating the HPGFC system with a Pullulan Standard Curve

Stock solutions of the pullulan standards were prepared at 12.5 mg/mL in water by bringing 25 mg of each pullulan to 2 mL. These were 0.22 µm filtered and frozen in 275 µL aliquots at –20 °C for later use. The largest pullulan standard, 708 kDa, required heating at 60 °C for 15 min for visual solubilization. The pullulan stocks were diluted to 2.5 mg/mL with buffer to match the mobile phase, and 10 µL of each standard was injected into the HPGFC system for analysis. The earliest eluting pullulan standard of 708 kDa visually diverged from linearity (Fig. 1, left panel). Because the size (Table 1) is well beyond the SuperMultipore PW-N exclusion limit specification of 100–150 kDa for ideal linear polymers such as polyethylene glycol (PEG), its elution volume was used as the void volume peak (Vo). Pullulan size on a log scale was plotted against elution volume (Ve) (Fig. 1, left panel), and the logarithm of pullulan molecular weight was plotted against Ve/Vo (Fig. 1, right panel). Based on these graphs, we excluded the 344 kDa and 320 Da data points for the final linear standard curve (Fig. 2). These values represent the outer edge of the molecular weight separation range for the SuperMultipore PW-N, using idealized polymers such as PEG. The mass of 344 kDa is much greater than the high mass limit of 100 kDa, so this is not surprising that its elution volume began to deviate from linear. However, most interestingly, the 320 Da standard was within (although at the edge of) the recommended mass separation range but

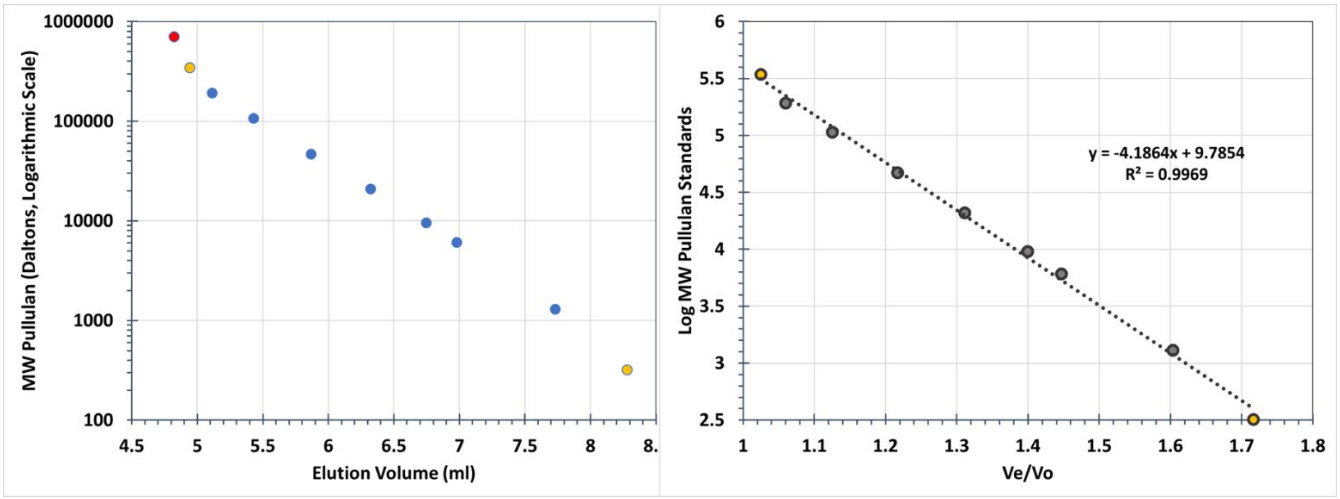

Figure 1—HPGFC showing the elution volume of all pullulan standards as a function of molecular weight (left panel). The red data point represents the 708 kDa pullulan standard and was used in the standard curve calculation as Vo (void peak). Utilizing the accepted sizing column calculations, a best fit line with a correlation coefficient (R2) of 0.997 resulted. It was clear from the data that the 320 Da and the 344 kDa peaks were deviating from ideal linear (right panel). Points in yellow were excluded for the final standard curve linear equation calculation (Fig. 2).

Table 1—Pullulan standard molecular weight, retention times, elution values, and Ve/Vo

	Pullulan category number	Pullulan MW std (kDa)[d]	Log MW	RT (min)	Elution volume (ml)	Vo or Ve	Ve/Vo
Void[a]	P-800	708	5.850033	4.822	1.928	Vo	—
Exclude[b]	P-400	344	5.536558	4.941	1.976	—	—
These values used for calibration	P-200	194	5.287802	5.11	2.044	Ve	1.059726
	P-100	107	5.029384	5.426	2.170	Ve	1.125259
	P-50	47.1	4.673021	5.867	2.346	Ve	1.216715
	P-20	21.1	4.324282	6.322	2.528	Ve	1.311074
	P-10	9.6	3.982271	6.746	2.698	Ve	1.399005
	P-5	6.1	3.78533	6.977	2.790	Ve	1.44691
	53168[c]	1.3	3.113943	7.73	3.092	Ve	1.603069
Exclude[c]	57191[c]	0.32	2.50515	8.278	3.311	—	—

[a]Value was used as the void peak to define Vo.
[b]Values were excluded for calculating the standard curve because of deviation from visual linearity.
[c]All standards were purchased from Shodex except Sigma Fluka Cat. No. 53168 and 57191.
[d]Molecular Weight Mode = Mp.

nonetheless showed deviation from linearity. The 194 kDa standard was greater than the recommended range by approximately 100 kDa but maintained the linearity. An upper limit of about 200 kDa is double the separation range for the SuperMultipore column when calibrated using PEG. For both of these outliers, the best explanation for why standard outcomes differ from the manufacturer's specifications is that the PEG and pullulans (as representing xylan) are comparable only in that they are soluble polymers. Polyethylene glycol is composed of ethylene glycol (EG), which has a monomer molecular weight of only 62 Da. Pullulan is composed of glucose with the molecular weight of 180.15 Da, approximately three times the mass of EG. This difference results in large polymer length differences when comparing standards of the same molecular weight and explains why a 194 kDa molecular weight pullulan standard elution extends the linear separation range of this column. This difference also explains why the 320 Da (a likely disaccharide) is effectively too small even though it is within the reported separation range of the SuperMultipore PW-N column. The 320 Da molecular weight is roughly equivalent to a PEG with a DP of 6, indicating that the molecule is sizably longer than a disaccharide.

Preparation of a Beechwood Glucuronoxylan Fraction for Xylanase Studies

Initial studies concerning the fractionation of BWX utilized a FPLC medium-pressure chromatography system with the SuperMultipore column. This FPLC chromatography

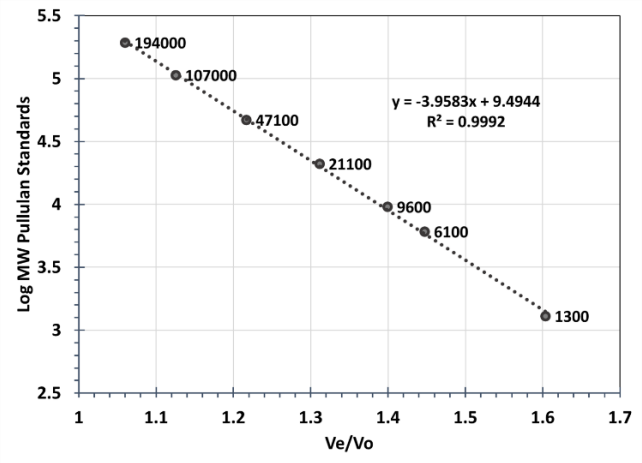

Figure 2—Final pullulan standard curve used for HPGFC sizing of BWX fractions. For a peak of unknown size run on this system, the size of the peak in Da is solved by inserting the unknown's Ve/Vo ratio into the following equation: size(Da) = $10^{-3.9583(Ve/Vo)+9.4944}$.

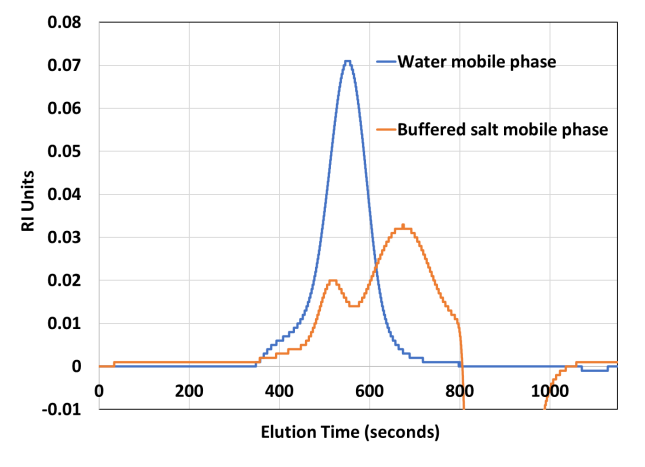

Figure 3—Initial elution observations of BWX in water (blue) compared to electrostatic interaction masking GFB (orange), showing how the single large water elution peak becomes two peaks when electrostatic interactions between the BWX and chromatography resin are masked with a high ionic strength mobile phase. The visually trimmed negative peak (orange) is thought to be the elution of water (an injection peak) in the high ionic strength buffer background.

system is capable of HPGFC but does not allow managed injections less than 100 µL, which was required for the planned approach. Using this system for initial observations, we injected 25 µL of 5 mg/mL BWX (125 µg) with water as a mobile phase to observe elution characteristics following the RI signal. In this study, a single large peak was obtained. The same injection was administered, but this time using GFB as mobile phase to mask electrostatic interactions, and this resulted in two peaks (Fig. 3). It was not clear how these two peaks differed, but it is possible that the left-most,

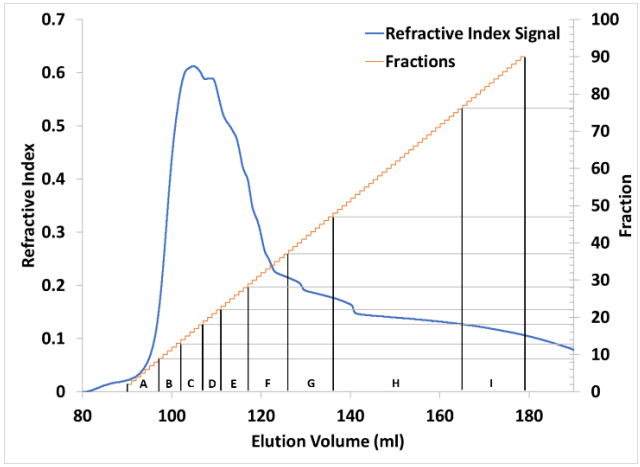

Figure 4—A volume of 5 mL of 20 mg/mL BWX (100 mg) was injected on a HiPrep 26/60 Sephacryl S-200HR. This was repeated six times and consecutive run fractions overlaid. Fraction groups were chosen as marked with red lines (A–I).

larger molecular mass peak consists of an agglomeration of xylan molecules, and the right-most, smaller molecular mass, consists of a more idealized soluble form of xylan. This result shows that a mixed population of xylan in water associates with itself and the HPGFC solid phase resin through ionic interactions. Because glucuronoxylans from hardwoods are substituted with glucuronic acid (GA) in a random manner (Jacobs et al. 2001), it is conceivable that the xylan with low GA is less soluble and tends to condense together similar to cellulose, whereas sections containing higher GA levels are expected to maintain greater solubility. This may result in the resolution of the two peaks.

Not knowing which BWX peak would serve the HPGFC method best, we opted to perform a simple water mobile phase elution on the FPLC using a preparative HiPrep 26/60 Sephacryl S-200HR (Fig. 4). We injected 5 mL samples of 20 mg/mL BWX and collected the fractions. From the single large peak resulting from six injections, sections were combined into groups (Fig. 4, grouped fractions A–I) with the idea to select a narrow peak fraction for HPGFC studies. These fractions were concentrated using a rotary evaporator, and their carbohydrate concentrations were determined using the phenol sulfuric acid assay (Table 2).

These concentrated Sephacryl S-200HR BWX consecutive peak sections were subsequently injected onto the SuperMultipore PW-N HPLC-based HPGFC system with the planned GFB mobile phase to consider their molecular weight characteristics. These fractions all resolved into two distinct peaks. Fractions A–G contain large amounts of both peaks with the left peak decreasing and the right peak increasing as consecutive peak sections progress from A–G. Figure 5 (left panel) shows each grouped fraction that was analyzed on the HPGFC system. The right panel of Figure 5 plots the calculated molecular weight of the right peak from the HPGFC analysis, showing how the latter fractions

Table 2—Guide to Sephacryl S-200HR water elution of BWX fraction grouping

Group[a]	Fractions	Grouped fraction approx. volume (ml)	Final volume (ml)[b]	Final conc. (mg/ml)	Total carbo-hydrate (mg)
A	11–88	43	1.90	3.56	6.76
B	9–12	24	2.56	8.40	21.50
C	13–17	29	3.47	14.79	51.32
D	18–21	24	2.83	14.06	39.79
E	22–27	36	3.69	13.01	48.01
F	28–36	52	3.55	12.76	45.30
G	37–46	59	3.68	9.05	33.30
H	47–75	175	4.67	17.09	79.81

[a]Fraction I was lost in handling.
[b]Grouped fractions were concentrated using a rotovap to the indicated volumes.

collected from the Sephacryl S-200HR column (Fig. 4) consisted of smaller molecular weight populations of xylan. From these studies, we decided to move forward with HPGFC development using grouped fraction H because it appeared to be of a more uniform composition than fractions A–G, which consist mostly of two distinct molecular weight species.

HPGFC Analysis of Xylanase Processed Beechwood Glucuronoxylan

This HPGFC system was further tested by digesting the selected BWX fraction (fraction H, ~16 kDa) with xylan-active enzymes of known function. *Pb*Xyn10A from *Paenibacillus* sp. JDR-2 is a well-characterized GH10 endoxylanase and is considered to be an ideal endo-acting enzyme (St John et al. 2006, 2012). When mixed with BWX, this endoxylanase will randomly bind to accessible sections of the β-1,4-xylan chain and hydrolyze. On average, a cleavage event is more likely to occur within the xylan chain, away from the ends of the polysaccharide, and therefore an endo-activity will be observed. *Ac*Xbh30A from *Acetivibrio clariflavus* is a well-characterized, high-specificity xylobiohydrolase (Crooks et al. 2021, St John et al. 2022), which removes xylobiose (the disaccharide hydrolysis product of xylan) from the nonreducing terminus of the xylan chain. This chain-end activity is an exo-activity. The endo- and exo-activities represent functional opposites with respect to how a xylan chain can be degraded. This can be readily identified using the HPGFC system. Hydrolysis using an endo-functioning xylanase will result in a decrease in molecular weight of the bulk xylan, which can be monitored by the rightward shift (molecular weight decrease) of the xylan peak. Conversely, an exo-activity, as exemplified using a xylobiohydrolase, will result in much slower rightward movement of the primary xylan peak but will yield xylobiose (approximately 280 Da).

Treatment of BWX Fraction H with *Pb*Xyn10A resulted in shifting of the peak slowly to the right, indicating that the xylan polymers were becoming smaller from endoxylanase activity (Fig. 6). In this case, endo-cleavage changed the size of the bulk xylan polymer, and as a result we observed a shift of this peak in the HPGFC result. The elution volumes (Table 3) allow tabulation of the changing molecular weight of the peak. When the reaction is allowed to process for a full hour with 100× higher enzyme concentration, smaller products do start to accumulate as the reaction gets closer to completion.

In contrast, when the same substrate is treated with the nonreducing-terminal xylobiohydrolase *Ac*Xbh30A, the main peak of Fraction H does not shift significantly when digested with 0.5 µg/mL enzyme, because the polymer size only becomes marginally smaller from the exo-activity (Fig. 7). Furthermore, a peak at 3.336 mL elution volume

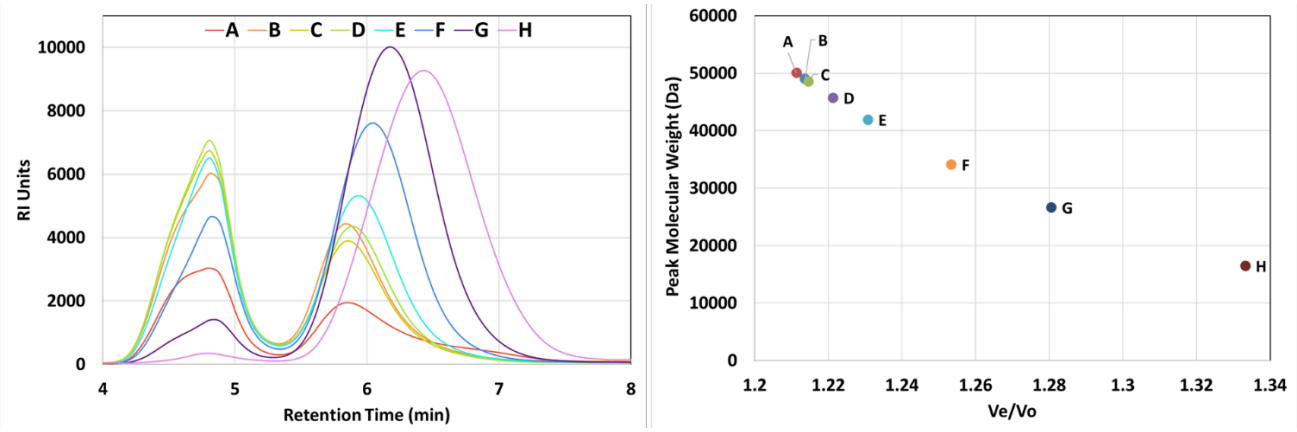

Figure 5—HPGFC of the HiPrep 26/60 Sephacryl S-200HR elution of BWX in a water mobile phase. The combined fraction groups (Fig. 4) were concentrated (Table 2) and injected on the HPGFC system. The elution time of the right-most smaller molecular weight component visually varied between the consecutive Sephacryl group fractions,so the calculated molecular weights were plotted as a function of the elution volume (Ve/Vo) (right panel). Sephacryl combined fraction H was used for enzyme studies. Its elution volume sized the xylan content at approximately 16 kDa.

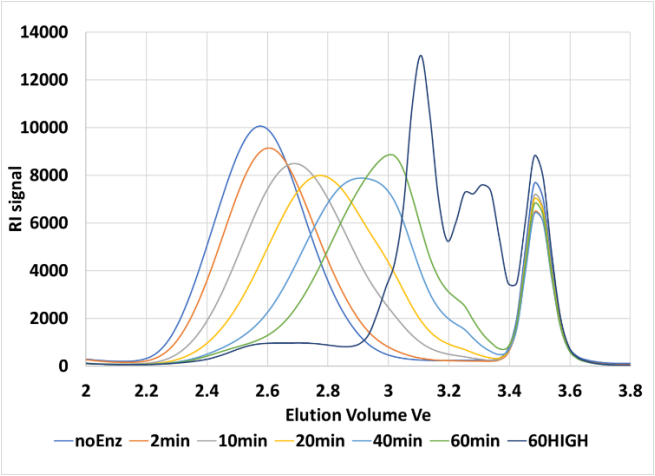

Figure 6—HPSEC traces for PbXyn10A endoxylanase digestion on BWX fraction H. All timepoints except 60HIGH used 0.5µg/mL PbXyn10A, 60HIGH used 50 ug/ml (100× greater enzyme).

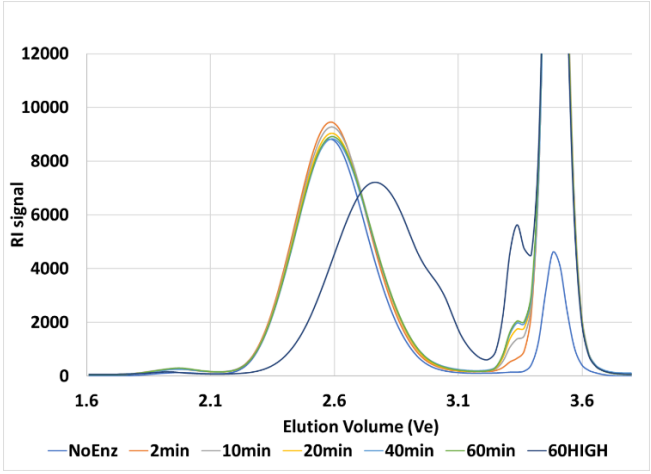

Figure 7—HPSEC RI traces for AcXbh30A xylobiohydrolase, an exoxylanase, on BWX Fraction H. All timepoints except 60HIGH used 0.5µg/mL AcXbh30A; 60HIGH used 50 µg/mL.

Table 3—BWX Fraction H peak mobility resulting from *Pb*Xyn10A endoxylanase treatment

*Pb*Xyn10A reaction		RT (min)	Ve (ml)	Ve/Vo	MW (Da)
No enzyme		6.440	2.576	1.335	16140
0.5 ug/ml	2 min	6.511	2.604	1.350	14113
	10 min	6.722	2.688	1.394	9472
	20 min	6.935	2.774	1.438	6332
	40 min	7.242	2.896	1.501	3545
	60 min	7.517	3.006	1.558	2108
50 ug/ml	60 min high, peak 1	7.763	3.105	1.609	1324
	60 min high, peak 2	8.150	3.260	1.690	637
	60 min high, peak 3	8.302	3.321	1.721	478

Table 4—BWX Fraction H peak mobility resulting from *Ac*Xbh30A exoxylanase treatment

*Ac*Xbh30A reaction		RT (min)	Ve (ml)	Ve/Vo	MW (Da)
No enzyme		6.549	2.583	1.339	15571
0.5 ug/ml	2 min	6.464	2.585	1.340	15424
	10 min	6.473	2.589	1.342	15164
	20 min	6.474	2.589	1.342	15136
	40 min	6.478	2.591	1.343	15022
	60 min	6.479	2.591	1.343	14993
50 ug/ml	60 min high, peak 1	6.912	2.764	1.433	6614
	60 min high, peak 2	8.342	3.336	1.729	443

appears, which is most likely the small xylobiose product with the molecular weight of approximately 282 Da. The calculated molecular weight at the 3.336 mL elution volume is 443 Da. This slight difference is likely due to the data point being off the linear range of the pullulan standard equation and also because xylan is not an exact equivalent to pullulan. The accumulation of xylobiose exemplifies the exo-activity of AcXbh30A, its effect on the xylan chain, and how such results can be studied by HPGFC.

Treatment of the BWX fraction H with a 100× increased *Ac*Xbh30A and digestion for a full hour confirmed previous data (Crooks et al. 2021) that *Ac*Xbh30A has a low-level endo-activity. The extent to which the primary bulk peak shifted to the right and the much larger yield of the xylobiose product verify the endo-activity. Table 4 indicates the bulk xylan peak shifted to 6.6 kDa with this aggressive xylobiohydrolase treatment.

Conclusion

The research reported here develops a laboratory methodology for the molecular weight-sizing of hemicellulosic polysaccharides such as xylans and glucomannans by HPGFC. Using the described system, data can be obtained revealing how xylanases (or mannanases) interact with a xylan (or mannan) polymeric substrate and therefore reveal how best to utilize the enzymes in bioprocess design. This system will be utilized to characterize enzymes suspected of acting processively and also to characterize and quantify background endo-hydrolysis occurring by a native xylobiohydrolase. This technique will also be developed further for the characterization of soluble and agglomerated woody biomass derived polysaccharides.

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